Journal of Microbiological Methods 84 (2011) 52–60

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

# Isolation of previously uncultured rumen bacteria by dilution to extinction using a new liquid culture medium

Nikki Kenters, Gemma Henderson, Jeyamalar Jeyanathan, Sandra Kittelmann, Peter H. Janssen *

*Grasslands Research Centre, AgResearch Ltd., Palmerston North 4442, New Zealand*

ARTICLE INFO

*Article history:*
Received 5 August 2010
Received in revised form 14 October 2010
Accepted 17 October 2010
Available online 26 October 2010

*Keywords:*
Bacteria
Growth medium
Identification
Isolation
Rumen

ABSTRACT

A new anaerobic medium that mimics the salts composition of rumen fluid was used in conjunction with a dilution method of liquid culture to isolate fermentative bacteria from the rumen of a grass-fed sheep. The aim was to inoculate a large number of culture tubes each with a mean of <1 culturable cell, which should maximize the number of cultures that develop from a single bacterium. This minimizes the effort that has to be put into purifying the resultant cultures. Of 1000 tubes, 139 were growth positive. Of the 93 that were able to be subcultured, 54 (58%) appeared to be pure cultures. The phylogenetic placements of these 54 cultures, together with another 6 cultures obtained from a preliminary study, were determined. Using a criterion of <93% 16S rRNA gene sequence identity to a previously named bacterium as a proxy for defining a new genus, 27 (45%) of the 60 cultures belonged to 14 potentially novel genera. Many of these had 16S rRNA genes that shared >97% sequence identity to genes of uncultured bacteria detected in various gastrointestinal environments. This strategy has therefore allowed us to cultivate many novel rumen bacteria, opening the way to overcoming the lack of cultures of many of the groups detected using cultivation-independent methods.



## 1. Introduction

The rumen contains a mixed community of microorganisms that ferments the feed ingested to products that are more readily utilized by the host animals. Instead of directly degrading plant material in the digestive tract, microbial degradation of the feed in the rumen, a fore-gut modification, results in the formation of volatile fatty acids that are absorbed across the rumen wall for use by the ruminant host (Hungate, 1966). In addition, microbial protein that is formed in the rumen is an important source of dietary nitrogen for the host animal. This complex interaction with the rumen microbes allows ruminants to feed on plant material rich in cellulose and hemicelluloses, which are poorly used by the animal itself, and so exploit a food source especially abundant in natural grasslands. Humans have domesticated some of these animals, and exploit the microbially mediated conversion of low value plant material to yield ruminant products like meat, wool, milk, and leather.

The microbial community that carries out the fermentation is made up of bacteria, archaea, fungi, and protozoa. Much of the primary attack on the plant material ingested by the animal is mediated by bacteria. The majority of these bacteria belong to only four phyla: *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Spirochaetes* (Firkins and Yu, 2006). Krause and Russell (1996) surmised that the species diversity of rumen bacteria was much higher than the 22 species described as being dominant when they reviewed the literature in 1996. Surveys of the 16S rRNA genes of bacteria in the rumen of different animals have since revealed a vast diversity of bacterial genera and species that have not been characterized, largely because there are no cultured representatives (e.g., Brulc et al., 2009; Edwards et al., 2004; Firkins and Yu, 2006; Kocherginskaya et al., 2001; Koike et al., 2003; Larue et al., 2005; Nelson et al., 2003; Ozutsumi et al., 2005; Ramsak et al., 2000; Sundset et al., 2007; Tajima et al., 1999, 2007; Yang et al., 2010a,b). The roles of most of these bacteria in the fermentation that occurs in the rumen remains unknown, and so we still lack a complete understanding of this economically and socially important microbial system. Foundation researchers developed media and methods for cultivating rumen bacteria, many of which were reviewed by McSweeney et al. (2005). A wide range of rumen bacteria has been isolated using these and other methods (Stewart et al., 1997), but, until recently, the lack of simple tools for classifying the isolates limited identification of phylogenetically novel isolations among the collections obtained. The advent of routine gene sequencing technologies and the availability of large public databases for comparative analysis have allowed rapid identification of new bacterial isolates on the basis of their 16S rRNA gene sequences (Clarridge, 2004).

One of the difficulties in studying the rumen bacterial community is the sensitivity to oxygen that the majority of the species exhibit. Methods have been developed that allow the isolation and manipulation of pure cultures of rumen bacteria, using closed vessels under

* Corresponding author. Tel.: +64 6 351 8300; fax: +64 6 351 8003.
*E-mail address:* peter.janssen@agresearch.co.nz (P.H. Janssen).

0167-7012/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2010.10.011

oxygen-free gas mixes, and artificial media that provide the growth requirements of the bacteria (Plugge, 2005; Sonnenwirth, 1972). These processes require some degree of skill and experience, and anyone who has isolated pure cultures of anaerobes appreciates the much slower progress and greater effort required compared to isolating most aerobes. Since the diversity of rumen bacteria is large, isolation of large numbers of cultures is especially daunting. Separation of cells of the different species that might grow, so that pure cultures can be derived, is not straight forward, since plating on gel surfaces is more difficult when working under anoxic conditions, although not impossible. If liquid cultures are used instead of solidified media, the species in a mixed inoculum that is best able to grow on that medium will dominate the culture, and will be repeatedly isolated, at the expense of species that are not pre-adapted to rapid growth in the microbiological medium.

In this study, we attempted to solve two of the problems facing microbiologists trying to isolate anaerobes from systems such as the rumen. Firstly, we developed a clear growth medium that chemically mimics the rumen milieu. Secondly, we used this medium in conjunction with a cultivation strategy that circumvented some of the problems of pure cultivation isolation of anaerobes, by attempting to avoid the enrichment of mixed cultures.

## 2. Materials and methods

### 2.1. Use of animals

The use of animals was approved by the AgResearch Grasslands Animal Ethics Committee and complied with the AgResearch Ltd. Code of Ethical Conduct for the Use of Animals in Research, Testing and Teaching, as prescribed in the Animal Welfare Act of 1999 and its amendments.

### 2.2. Medium preparation

A bicarbonate-buffered mineral medium supplemented with vitamins was developed and used to culture rumen bacteria. The mineral salts solution contained (in 950 ml of distilled water) 1.4 g of $KH_2PO_4$, 0.6 g of $(NH_4)_2SO_4$, 1.5 g of KCl, 1 ml of trace element solution SL10 (Widdel et al., 1983), 1 ml of selenite/tungstate solution (Tschech and Pfennig, 1984), and 0.4 ml of a 0.1% (w/v) resazurin solution. The mineral salts solution was mixed and then boiled under $O_2$-free 100% $CO_2$, before being cooled in an ice bath while it was bubbled with 100% $CO_2$. Once the mineral salts solution was cool, 4.2 g of $NaHCO_3$ and 0.5 g of L-cysteine·HCl·$H_2O$ were added per litre. The cooled and supplemented mineral salts solution was then dispensed into Hungate tubes (16 mm dia., 125 mm long; Bellco Glass, Vineland, NJ, USA), while being gassed with 100% $CO_2$, at 9.5 ml of medium per tube. The tubes were closed with black butyl rubber seals and perforated plastic caps. This gave an initial headspace of 100% $CO_2$ in these tubes. These tubes were sterilized by autoclaving for 20 min at 121 °C. The tubes containing the medium, which was designated RM02, were stored in the dark for at least 24 h before use. When required, 0.5 ml of GenRFV (see section 2.3) was added per 9.5 ml of RM02. The final pH was 6.5.

### 2.3. Preparation of rumen fluid and substrates

Rumen contents were collected from two ruminally fistulated cows that had been fed pasture hay for 48 h after being on a rye-grass clover pasture. The cows were dry female Friesian crosses, 8–10 years old, and weighed about 570 kg each. Feed was withheld from the animal after 4 pm and rumen contents collected the next day at 9:30 am. The material was filtered through a double layer of cotton cheesecloth with a mesh size of approx. 1 mm (Stockinette; Cirtex Industries Ltd., Thames, New Zealand), and then fine particulate material was removed from the liquid fraction by centrifugation at 10,000 *g* for 20 min. The supernatant (rumen fluid) was stored frozen at −20 °C. The rumen fluid was thawed before use, and any new precipitates that formed were removed by centrifugation at 12,000 *g* for 15 min. The supernatant was retained and bubbled for 10 min with 100% $N_2$ gas, before inactivating viruses by autoclaving for 15 min in partly filled serum vials closed with blue butyl rubber stoppers and aluminium closures (20 mm diameter; Bellco) under 100% nitrogen. The anoxic conditions are to limit the formation of unwanted oxidation products during autoclaving. The autoclaved rumen fluid was then stirred under air, and 1.63 g of $MgCl_2 \cdot 6H_2O$ and 1.18 g of $CaCl_2 \cdot 2H_2O$ added per 100 ml. This formed a heavy precipitate, which was removed by centrifuging at 30,000 *g* and 4 °C for 60 min. The supernatant is the clarified rumen fluid. To each 100 ml of clarified rumen fluid were added: 0.34 g of D-glucose, 0.34 g of D-cellobiose, 0.30 g of D-xylose, 0.30 g of L-arabinose, 0.88 ml of Na L-lactate syrup (50% in water), 2 g of casamino acids, 2 g of Bacto-peptone and 2 g of yeast extract. This was well mixed and then bubbled with $N_2$ gas for 15 min, before being transferred through a 0.22 μm pore size Millex GP sterile filter (Millipore Corp., Bedford, MA, USA) via a sterile syringe and needle into a sterile $N_2$-flushed serum vial sealed with a blue butyl rubber stopper and an aluminium closure (Bellco). Two millilitres of Vitamin 10 concentrate (see section 2.4) was then added per 100 ml of preparation by syringe and needle. This clarified rumen fluid containing substrates and vitamins was designated GenRFV.

### 2.4. Vitamin 10 concentrate

Vitamin 10 concentrate contained 1000 ml of distilled water, 40 mg of 4-aminobenzoate, 10 mg of d-(+)-biotin, 100 mg of nicotinic acid, 50 mg of hemicalcium D-(+)-pantothenate, 150 mg of pyridoxamine hydrochloride, 100 mg of thiamine chloride hydrochloride, 50 mg of cyanocobalamin, 30 mg of D,L-6,8-thioctic acid, 30 mg of riboflavin and 10 mg of folic acid. After preparation, the solution was well mixed and then bubbled with $N_2$ gas for 15 min, before being transferred through a 0.22-μm pore size Millex GP sterile filter (Millipore) using a sterile syringe and needle to a sterile sealed $N_2$-flushed serum vial (Bellco).

### 2.5. Rumen samples for cultivation

All samples were collected from the same ruminally fistulated wether sheep (Romney cross, 9 years old, 80 kg). This animal was kept at the AgResearch Grasslands Campus, and allowed to feed *ad libitum* on a rye-grass clover pasture. Samples of total rumen contents were collected via the fistula on 4 different occasions. All sampling was conducted at 9.30 to 10.30 am, within 1 h of the animal being taken from the pasture. Samples were collected in 450-ml glass containers with screw-top sealable metal lids. The containers were gassed with $CO_2$ prior to use, and filled to the top with sample, before being capped and brought to the laboratory for use within 30 min of collection.

### 2.6. Cultivation of bacteria

Forty grams of rumen contents from the sheep were blended in 360 ml of RM02 under anoxic conditions using a Waring blender (Waring Products Inc., Torrington, CT, USA) with a glass chamber and tight-fitting rubber lid on the HI setting for four bursts of 20 s each, with an interval of 30 s between each burst. The glass vessel was gassed with $CO_2$ prior to use and during subsequent manipulations. Using a 16-gauge hypodermic needle and a sterile plastic syringe, 1 ml was transferred from the blender into a 150 ml serum bottle sealed with a blue butyl rubber stopper and an aluminium closure (Bellco) and containing 99 ml of RM02 supplemented with 5% (vol/vol) of GenRFV under a gas phase of 100% $CO_2$. Further serial dilutions were